

REVISED VERSION

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 November 2000 (23.11.2000)

PCT

(10) International Publication Number  
WO 00/70079 A1

(51) International Patent Classification<sup>7</sup>: C12Q 1/00, 1/44,  
G01N 31/00, 33/52, 33/53, 33/58, 33/566, 33/577

(21) International Application Number: PCT/US00/12863

(22) International Filing Date: 10 May 2000 (10.05.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/311,797 13 May 1999 (13.05.1999) US

(71) Applicant: ORTHO-MCNEIL PHARMACEUTICAL,  
INC. [US/US]; US Route 202, Raritan, NJ 08869 (US).

(72) Inventors: ALLAN, George, F.; 18 Fawn Run, Trenton,  
NJ 08806 (US). CLANCY, Joanna; 41 Avalon Road, Tren-  
ton, NJ 08638 (US). HUTCHINS, Amy; 1821 Springtown  
Road, Alpha, NJ 08865 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,  
DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU,  
ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,

LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ,  
PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT,  
TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent  
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent  
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,  
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- With international search report.
- Before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments.

(88) Date of publication of the revised international search  
report: 8 February 2001

(15) Information about Correction:  
see PCT Gazette No. 06/2001 of 8 February 2001, Section  
II

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: HIGH THROUGHPUT ASSAY FOR MODULATORS OF NUCLEAR RECEPTORS

(57) Abstract: Herein we describe the design and use of a scintillation proximity-based assay for screening compounds for estrogen receptor binding. The estrogen receptor exists in two forms,  $\alpha$  and  $\beta$ , and is a target for preventing osteoporosis and other post-menopausal conditions, and for treating breast cancer. The methods described herein can easily be adapted for use with other nuclear receptors where at least one ligand is known.

WO 00/70079 A1

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/12863

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbol(s))

U.S. : Please See Extra-Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST and WEST Patent Database, STN-biosis, caplus, haplus, medline.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,783,398 A(MARCY et al.) 21 July 1998, see entire document.	1-5
Y	US 5,512,429 A (WILTON) 30 April 1996, see full document.	1-5
Y	US 4,568,649 A (BERTOGLIO-MATTE) 04 February 1986, see full document.	2-5
Y	US 5,389,517 A (WOTIZ et al) 14 February 1995, see entire document.	2-5



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

20 JUNE 2000

Date of mailing of the international search report

01 DEC 2000

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

LISA V. COOK

Telephone No. (703)305-3399



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12Q 1/00, 1/44, G01N 31/00, 33/52, 33/53, 33/54, 33/58, 33/566, 33/577</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/70079</b> <b>(43) International Publication Date:</b> 23 November 2000 (23.11.00)
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<b>(54) Title:</b> HIGH THROUGHPUT ASSAY FOR MODULATORS OF NUCLEAR RECEPTORS  <b>(57) Abstract</b>  Herein we describe the design and use of a scintillation proximity-based assay for screening compounds for estrogen receptor binding. The estrogen receptor exists in two forms, $\alpha$ and $\beta$ , and is a target for preventing osteoporosis and other post-menopausal conditions, and for treating breast cancer. The methods described herein can easily be adapted for use with other nuclear receptors where at least one ligand is known.		

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TITLE OF THE INVENTIONHIGH THROUGHPUT ASSAY FOR MODULATORS OF NUCLEAR  
5 RECEPTORSBACKGROUND OF THE INVENTION

Members of the steroid, thyroid hormone, retinoid and peroxisome  
10 proliferator-activated receptor superfamily are collectively known as nuclear  
receptors and play diverse roles in mammalian physiology (Tsai, M. J. and O'Malley,  
B. W. (1994). *Ann Rev Biochem* 63, 451-486). For this reason, and because nuclear  
receptors are natural receptors for small lipophilic molecules, they are attractive  
therapeutic targets for the pharmaceutical industry, yielding some of the most  
15 important pharmaceuticals available, including oral contraceptives, anti-cancer drugs,  
anti-asthmatic compounds, anti-diabetic compounds and skin treatments. Given the  
intense interest in these therapeutic areas, these receptors will likely be active  
pharmaceutical targets for the foreseeable future. Moreover, the size of the nuclear  
receptor superfamily has greatly expanded, so that there are at least 50 known  
20 mammalian members, the majority of which lack a known ligand. It is likely that, as  
ligands for these so-called orphan receptors are identified, many will represent  
equally fruitful drug targets. A robust, automated screening protocol will be required  
for screening compounds against this family of receptors. Herein we describe a  
method to screen ligands for one of these receptors, the estrogen receptor. The  
25 method is rapid, robust, reliable, can be used in an ultra-high throughput robotic  
screen, and is adaptable for use with any known nuclear receptor.

In order to be technically feasible and to maintain reasonable experimental variability, high throughput robotic screens require assays that are durable and simple. Current experimental methods to detect ligand/nuclear receptor interactions, including

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competitive binding assays relying on separation of bound ligand from free by adsorption or filtration methods, are not technically feasible for high throughput screens due to the degree of sample manipulation that is required. A homogeneous assay, where binding and detection are performed in the same vessel, minimizes sample manipulation and thus experimental variability.

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Using scintillation proximity technology, homogeneous assays have been developed for a variety of molecular targets (Cook, N. D. (1996). *Drug Discovery Today* 1: 287-294; Picardo, M. and Hughes, K.T. (1997). In *High Throughput Screening* [Devlin, J. P. (Ed)], Dekker, New York, NY, pp. 307-316). Briefly, the target of interest is immobilized either by coating or incorporation on a solid support that contains a fluorescent material. A radioactive molecule, brought in close proximity to the solid phase by associating with the immobilized target, causes the fluorescent material to become excited and emit visible light. Emission of visible light forms the basis of detection of successful ligand/target interaction, and is measured by an appropriate monitoring device. An example of a scintillation proximity assay is disclosed in United States Patent No. 4,568,649, issued February 4, 1986. US patent 5,770,176 describes assays for nuclear receptors wherein the functional receptor binds to immobilized nucleic acid. Materials for these types of assays are commercially available from DuPont NEN® (Boston, Massachusetts) under the trade name FlashPlate™. This methodology has been applied to membrane proteins, transcription factors and antibodies. Recently, it has also been applied to nuclear receptors (Haggbblad, J., Carlsson, B., Kivela, P. and Siitari, H. (1995). *BioTechniques* 18: 146-151). In this example, the authors adsorbed recombinant

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estrogen receptor non-specifically to the vessel walls. Another example of nuclear receptor use in proximity assay is described using the peroxisome proliferator-activated receptor  $\gamma$  (Elbrecht, A., Chen, Y., Adams, A., Berger, J., Griffin, P., Klatt, T., Zhang, B., Menke, J., Zhou, G., Smith, R.G., Moller, D. E. (1999) *J. Biol Chem* 274 (12) 7913-7922., Nichols, J. S., Parks, D.J. Consler, T. G., Blanchard, S.G. (1998). *Anal Biochem*

257(2) 112 - 119) Herein we will describe a version of the assay where an anti-receptor antibody is used to specifically bind the receptor to the FlashPlate. This has the advantage of dramatically reducing the amount of receptor required for a screen. The method can be applied to any nuclear receptor for which an antibody and ligand are available.

#### SUMMARY OF THE INVENTION

The present invention provides a high throughput method to detect ligands that bind to full-length or fragmented nuclear receptor proteins. The method of the invention is a robust, sensitive, non-perturbing assay. The present invention provides an assay of simple design such that it is easily automated and an assay design that can be easily modified to allow different nuclear receptor targets to be tested without significant modification of the design. The present invention relates to a scintillation proximity method to screen compounds that bind to nuclear receptor proteins by using displacement of an established radiolabeled ligand. In a most preferred embodiment a solution containing two ligands, one sample ligand and a radiolabeled ligand, are incubated with immobilized nuclear receptor protein for sufficient time to allow binding equilibrium to be achieved. Afterwards, the bound radiolabel is measured indirectly by monitoring the fluorescence of the immobilized support carrying the nuclear receptor protein. Therefore, measuring reduced fluorescence of a vessel containing a sample, the

radiolabeled ligand, and the nuclear receptor compared to a vessel containing only radiolabeled ligand and nuclear receptor would indicate that the sample was specifically bound to the nuclear receptor protein.

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Herein we describe the design and use of a scintillation proximity-based assay for screening compounds for estrogen receptor binding. The estrogen receptor exists in two forms,  $\alpha$  and  $\beta$ , and is a target for preventing osteoporosis and other post-menopausal conditions, and for treating breast cancer. The methods described herein can easily be adapted for use with other nuclear receptors where at least one ligand is known.

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#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 - **Establishment of a homogeneous competitive binding assay for estrogen receptor  $\alpha$  ligands using antibody capture of the receptor.** Estrogen receptor  $\alpha$  (0.75 or 10 pmol) was added in triplicate to the wells of 96 well FlashPlates previously coated with goat anti-mouse immunoglobulin. SRA-1010 anti-estrogen receptor  $\alpha$  antibody (0.5  $\mu$ g) was added or omitted as shown. The plates were incubated at approximately 4°C for three days. Then, excess protein was removed by washing the plates. Next, [ $^3$ H] estradiol and different concentrations of 17 $\beta$ -estradiol diluted in vehicle (0.75% [v/v] dimethyl sulfoxide) were added and incubated for one hour at ambient temperature prior to measurement using a TopCount scintillation counter. Data shown are averaged from two plates.

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Figure 2 - **Effect of different antibodies.** Estrogen receptor  $\alpha$  (0.75 pmol) and 0.5  $\mu$ g of commercially available anti-estrogen receptor  $\alpha$  antibodies were added in duplicate to the wells of a 96 well FlashPlate previously coated with goat anti-mouse



immunoglobulin, and incubated at approximately 4°C overnight. The next day, the excess protein was removed by washing the plate. Then [<sup>125</sup>I] estradiol, and either 500 nM 17β-estradiol (“+ competitor”) or the solvent vehicle (0.75% [v/v] dimethyl sulfoxide; “w/o competitor”), were added and incubated for 30 minutes at ambient temperature prior to measurement using a TopCount scintillation counter. The negative control for this assay includes all components except for the estrogen receptor (No receptor).

- 10 **Figure 3 - Stability of the reaction.** Estrogen receptor α and anti-estrogen receptor α antibody SRA-1010 were added in duplicate to the wells of a 96 well FlashPlate previously coated with goat anti-mouse immunoglobulin, and incubated at approximately 4°C overnight. The next day, the excess protein was removed by washing the plate. Then [<sup>125</sup>I] estradiol and either 500 nM 17β-estradiol (“+ competitor”) or the solvent vehicle (0.75% [v/v] dimethyl sulfoxide; “w/o competitor”), were added and incubated for various time points at ambient temperature prior to measurement using a TopCount scintillation counter.

**Figure 4 - The order of addition of receptor is critical for assay performance.**

- 20 Estrogen receptor α and anti-estrogen receptor α antibody SRA-1010 (“Receptor with antibody”) or anti-estrogen receptor α antibody SRA-1010 alone (“Receptor with tracer”) were added in duplicate to the wells of a 96 well FlashPlate previously coated with goat anti-mouse immunoglobulin, and incubated at approximately 4°C overnight. The next day, the excess protein was removed by washing the plate. Then [<sup>125</sup>I] estradiol (“Receptor with antibody”) or [<sup>125</sup>I] estradiol with estrogen receptor α (“Receptor with tracer”) were added and incubated for 30 minutes at ambient temperature prior to

measurement using a TopCount scintillation counter. The negative control for this assay includes all components except for the estrogen receptor (No receptor).

- 5     **Figure 5 - Saturation binding analysis.** Estrogen receptor  $\alpha$  and anti-estrogen receptor  $\alpha$  antibody SRA-1010 were added in sextuplicate to 96 well FlashPlates previously coated with goat anti-mouse immunoglobulin, and incubated at approximately 4°C for three days. Excess protein was removed by washing the plates. Then different concentrations of [ $^3$ H] estradiol were added in the presence of 1.6  $\mu$ M 17 $\beta$ -estradiol or
- 10    0.75% (v/v) dimethyl sulfoxide ("w/o competitor"). The plates were incubated for 1 hr at ambient temperature prior to measurement using a TopCount scintillation counter. Data are averaged from two plates.

**Figure 6 - Dose-dependent competition of binding of estradiol to estrogen receptor  $\alpha$ .**

- 15    Estrogen receptor  $\alpha$  and anti-estrogen receptor  $\alpha$  antibody SRA-1010 were added in triplicate to the wells of 96 well FlashPlates previously coated with goat anti-mouse immunoglobulin, and incubated at approximately 4°C for three days. Then, the excess protein was removed by washing the plates. Next, [ $^3$ H] estradiol and different concentrations of three different competitors were added and incubated for 1 hour at
- 20    ambient temperature prior to measurement using a TopCount scintillation counter. Data are averaged from two plates.

**Figure 7 - Use of a polyclonal antibody to generate a homogeneous competitive binding assay for ligands binding to estrogen receptor  $\beta$ .** Estrogen receptor  $\beta$  (1.7 pmol) and different amounts of anti-estrogen receptor  $\beta$  antibody PA1-313 were added in duplicate to the wells of a 96 well FlashPlate previously coated with sheep anti-rabbit immunoglobulin, and incubated at approximately 4°C overnight. The next

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day, excess protein was removed by washing the plate. Then [ $^3\text{H}$ ] estradiol, and either 500 nM 17 $\beta$ -estradiol (“+ competitor”) or the solvent vehicle (0.75% [v/v] dimethyl sulfoxide; “w/o competitor”), were added and incubated for one hour at ambient temperature prior to measurement using a TopCount scintillation counter.

Figure 8)- **Flow diagram of the binding assay.** For illustrative purposes, the assay of the present invention is shown using antibody capture as the affinity capture.

## 10 DETAILED DESCRIPTION

### DEFINITIONS:

The term “protein domain” as used herein refers to a region of a protein that can fold into a stable three-dimensional structure independently of the rest of the protein. This structure may maintain a specific function associated with the domain’s function within the intact protein, including enzymatic activity, creation of a recognition motif for another molecule, or provision of the necessary structural components for a protein to exist in a particular environment. Protein domains are usually evolutionarily conserved regions of proteins, both within a protein family and within other protein superfamilies that require similar functions.

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The term “protein superfamily” as used herein refers to proteins whose evolutionary relationship may not be entirely established or that may be distant by accepted phylogenetic standards, but that show a similar three-dimensional structure or display unique consensus critical amino acids.

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The term “fusion protein” as used herein refers to a protein construct that is the result of combining multiple protein domains or linker regions for the purpose of gaining function of the combined functions of the domains or linker regions. This is most often

accomplished by molecular cloning of the nucleotide sequences to result in the creation of a new polynucleotide sequence that codes for the desired protein. Alternatively, creation of a fusion protein may be accomplished by chemically joining two proteins  
5 together.

The term "linker region" or "linker domain" or similar such descriptive terms as used herein refers to stretches of polynucleotide or polypeptide sequence that are used in the construction of a cloning vector or fusion protein. Functions of a linker region can  
10 include introduction of cloning sites into the nucleotide sequence, introduction of a flexible component or space-creating region between two protein domains, or creation of an affinity tag for specific molecular interaction. A linker region may be introduced into a fusion protein without a specific purpose, but as a compromise that results from choices made during cloning.

15 The term "cloning site" or "polycloning site" as used herein refers to a region of the nucleotide sequence contained within a cloning vector or engineered within a fusion protein that has one or more available restriction endonuclease consensus sequences. The use of a correctly chosen restriction endonuclease results in the ability to isolate a  
20 desired nucleotide sequence that codes for an in-frame sequence relative to a start codon that yields a desirable protein product after transcription and translation. These nucleotide sequences can then be introduced into other cloning vectors, used to create novel fusion proteins, or used to introduce specific site-directed mutations. It is well known by those in the art that cloning sites can be engineered at a desired location by  
25 silent mutations, conserved mutation, or introduction of a linker region that contains desired restriction enzyme consensus sequences. It is also well known by those in the art that the precise location of a cloning site can be flexible so long as the desired function of the protein or fragment thereof being cloned is maintained.

As used herein, "expression vectors" are defined as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic or prokaryotic genes in a variety of hosts including *Escherichia coli*, blue-green algae, plant cells, insect cells, fungal cells including yeast cells, and animal cells.

As used herein, a "functional derivative" of the estrogen receptor is a construct that possesses a biological activity, either functional or structural, that is substantially similar to the properties described herein. The term "functional derivative" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" of the construct presented. The term "fragment" is meant to refer to any nucleic acid or polypeptide subset of the modules described herein. The term "variant" is meant to refer to a construct or coding sequence module substantially similar in structure and function to either the entire estrogen receptor molecule or to a fragment thereof. A construct is "substantially similar" to the estrogen receptor construct if both molecules have similar structural characteristics or if both molecules possess similar biological properties ie, can be manipulated such that they express recombinant estrogen receptor. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the entire estrogen receptor molecule or to a fragment thereof.

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The term "sample" as used herein in connection with a ligand for a nuclear receptor protein refers to an organic or inorganic molecule that has the potential to disrupt the specific binding of a known ligand for the receptor. For example, but not to

limit the scope of the current invention, samples may include small organic or inorganic molecules, synthetic or natural amino acid peptides, proteins, or synthetic or natural nucleic acid sequences, or any chemical derivatives of the aforementioned.

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The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule.

Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

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The term "radiolabeled ligand" as used herein in connection with displacement assays is a ligand known to bind to a known nuclear receptor, which is capable of being made radioactive. The types of radioactive tracers used to label the ligand include any of the known  $\beta$ -particle emitters or Auger electrons, including [ $^3\text{H}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], [ $^{33}\text{P}$ ], [ $^{32}\text{P}$ ], [ $^{125}\text{I}$ ], and [ $^{131}\text{I}$ ], with [ $^3\text{H}$ ] being generally preferred due to its relative safety. In a most preferred embodiment of the present invention, the concentration of radiolabeled ligand used is closely matched to the natural ligand's affinity ( $K_d$ ) for its receptor. Most natural ligands for nuclear receptors have  $K_d$ 's below 10 nanomolar. Radiolabeled nuclear receptor ligands are available from a number of commercial sources, including NEN Life Sciences and Amersham. Labeled ligands are available for the estrogen, progesterone, androgen, glucocorticoid, vitamin D, thyroid hormone and retinoic acid receptors.

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The term "receptor" as used herein refers to members of the nuclear receptor family or superfamily. Sources of receptor for use in the invention can include tissue or cell extracts, or purified receptor derived from natural or recombinant sources expressed in *E. coli*, yeast, insect or mammalian cells, or other cell source, with purified,

recombinant receptor being generally preferred. Crude tissue or cell extracts, or partially purified extracts, may also be appropriate, provided that the receptor is expressed at at least approximately 5 micromolar concentration in the extract. It is well known by those  
5 in the art that different receptors from different animal species may bind the same ligands, and this invention extends to all sources of nuclear receptors where binding of the mammalian ligand can be shown.

The term "high throughput" as used herein in connection with displacement  
10 assays refers to an assay design that allows easy analysis of multiple samples simultaneously, capacity for robotic manipulation, and small sample volume. Examples of assay formats include 96-well or 384-well plates used for liquid handling experiments. It is well known by those in the art that as miniaturization of plastic molds and liquid handling devices is advanced, or as improved assay devices  
15 are designed, greater numbers of samples may be analyzed using the design of the present invention. Use of 96-well plate assays in the examples is given for illustrative purposes only.

The present invention provides a high throughput method to detect ligands that  
20 bind to full-length or fragmented nuclear receptor proteins that is of simple design such that it is easily automated and can be easily modified to allow different nuclear receptor targets to be tested without significant modification. The present invention relates to a scintillation proximity method to screen compounds that bind to nuclear receptor proteins by using displacement of an established radiolabeled ligand. In a most preferred  
25 embodiment a solution containing two ligands, one sample ligand and a radiolabeled ligand, are incubated with immobilized nuclear receptor protein for sufficient time to allow binding equilibrium to be achieved. Afterwards, the bound radiolabel is measured indirectly by monitoring the fluorescence of the immobilized support carrying the nuclear

receptor protein. Therefore, measuring reduced fluorescence of a vessel containing a sample, the radiolabeled ligand, and the nuclear receptor compared to a vessel containing only radiolabeled ligand and nuclear receptor would indicate that the sample was  
5 specifically bound to the nuclear receptor protein. A schematic illustration is shown in Figure 8.

The present invention provides use of any scintillant-impregnated or coated high throughput vessel, with multiple well plates (96 well, 384 well *etc.*) being  
10 generally preferred. Uncoated scintillant-containing plates can be purchased from Packard or NEN Life Sciences. The plates can also be purchased pre-coated with an anti-species antibody (depending on the antibody to be used), or anti-species antibody can be pre-coated to a basic plate by the user prior to incubation with receptor and anti-receptor antibody. Where tagged proteins are to be used, plates can be purchased  
15 pre-coated with streptavidin, or appropriate capture material (anti-Flag antibody, anti-glutathione *S*-transferase antibody, *etc.*) can be pre-coated to plates prior to incubation with the tagged protein. For purposes of Histidine tagged fusion proteins, one could use a scintillation proximity vehicle coated with a nickel surface. Pre-coating of antibodies can be performed by overnight incubation at 4 °C in appropriate  
20 buffers, using plates containing anti-species antibody or treated with, for example, poly-lysine.

In order to be sufficiently sensitive, a scintillation proximity assay requires efficient immobilization of the protein of interest to the scintillant-containing surface.  
25 Binding of whole estrogen receptor can be achieved by simple adsorption, but this does not usually yield a stably bound protein. While larger amounts of receptor would increase the sensitivity of the reaction, this would create a greater requirement for purified receptor, and thus make the method less cost-effective. To overcome this



limitation, the protein target can be immobilized to the surface using an intermediate affinity capture motif. Methods of affinity capture include but are not limited to: biotin/avidin technology, directed fusion protein interactions such as between glutathione  
5 and glutathione *S*-transferase or dextran and maltose binding domain, and antibodies directed against the protein itself or a fragment thereof, or against a fusion tag contained in a fusion protein.

The type of antibody used to establish an assay of this type may be either  
10 monoclonal (recognizing one epitope of its target protein) or polyclonal (recognizing multiple epitopes). The optimal antibody for a given receptor can be determined experimentally, because some antibodies may yield greater signal-to-noise ratios than others, depending on the epitope or epitopes of the receptor that each recognizes. Monoclonal and polyclonal antibodies against nuclear receptors are available from a  
15 number of commercial sources (Affinity BioReagents, Stressgen, Transduction Laboratories). Custom antibodies can be generated by the user according to standard procedures (immunization of rabbits or mice with pure protein or peptide). The amount of antibody to be used per well will depend primarily on its avidity for the receptor or receptor fragment, and is well known by those in the art. Likewise, the positions of  
20 engineered affinity tags should be chosen so as not to disrupt ligand binding. However, for nuclear receptors in general, use of an epitope outside the ligand binding domain, or positioning of a tag amino-terminal of the ligand binding domain, is unlikely to lead to disruption.

25 The present invention allows use of any buffer that maintains an appropriate pH and salt concentration to allow receptor binding to the plate and for incubation of the receptor with the ligands. Depending on the stability of any specific nuclear receptor, the present invention allows buffer additives that enhance stability of a

particular protein, and these conditions are well known by those in the art. The estrogen receptor  $\alpha$ , which is a relatively unstable protein, uses high concentrations of dithiothreitol in a complex buffer, commercially available from Panvera. The  
5 estrogen receptor  $\beta$ , which is more stable, uses a simpler buffered salt solution, also commercially available from Panvera.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal  
10 inhibition of the binding of test ligand to the receptor, or modulation of its activity, while minimizing any potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The following examples illustrate the present invention without, however,  
15 limiting the same thereto.

#### EXAMPLE 1 - Materials

*Sources of receptor:* The examples taught herein use purified estrogen receptor  $\alpha$  or  $\beta$  from Panvera (Madison, WI, USA). The human estrogen receptor  $\alpha$  is expressed in  
20 baculovirus-infected insect cells and subsequently purified to greater than 95% purity by the manufacturer. Estrogen receptor  $\beta$  is expressed and purified in a similar way. For all methods described herein, approximately 50 ng (0.75 pmol) of purified estrogen receptor  $\alpha$  were added to each well. Approximately 100 ng (1.7 pmol) of estrogen receptor  $\beta$   
25 were added to each well.

*Sources of affinity capture intermediate:* The examples taught herein use commercially available antibodies, either monoclonal or polyclonal, with specific binding to the

matching receptor. For all methods described herein, approximately 500 ng to 2  $\mu$ g of antibody were added to each well. Anti-estrogen receptor  $\alpha$  mouse monoclonal antibodies tested were obtained as follows: MA1-310 from Affinity BioReagents (Golden, CO, USA), E51320 from Transduction Laboratories (Lexington, KY, USA), SRA-1000 and SRA-1010 from Stressgen Biotechnologies (Victoria, BC, Canada). The anti-estrogen  $\beta$  rabbit polyclonal antibody PA1-313 was obtained Affinity BioReagents (Golden, CO, USA).

- 10 *Sources of radiolabeled ligand and sample ligands:* Radiolabeled  $16\alpha$ -[ $^{125}$ I]-iodo-3, 17 $\beta$ -estradiol and [2,4,6,7,16,17- $^3$ H(N)]-estradiol were both obtained from NEN Life Science Products.

- 15 *Sources of plates:* The examples taught herein use scintillant-containing FlashPlate Plus<sup>®</sup> 96 well assay plates which have been pre-coated with either a goat anti-mouse immunoglobulin antibody (for estrogen receptor  $\alpha$ ) or a sheep anti-rabbit immunoglobulin antibody (for estrogen receptor  $\beta$ ), both from NEN Life Science Products (Boston, MA, USA). These plates could also be used to capture anti-Flag or anti-glutathione *S*-transferase antibodies, which in turn would capture Flag-tagged or
- 20 glutathione *S*-transferase-fused receptor or receptor fragments, respectively. Alternatively, streptavidin-coated plates (available from NEN) could be used to capture a biotinylated receptor or receptor fragment. One may also coat basic scintillation plates oneself with a capture agent. Basic plates are available from NEN and Packard. To
- 25 optimize binding, treatment of wells with poly-lysine prior to incubation with the capture agent can be performed.

*Diluents and wash solutions:* Estrogen Screening Buffer (Panvera) was used for estrogen receptor  $\alpha$  incubations. Dithiothreitol was added to 5 mM prior to use. BGG buffer (Panvera) was used for estrogen receptor  $\beta$  incubations. All washes were performed in  
5 phosphate-buffered saline, pH 7.2.

## EXAMPLE 2

### Establishment of a High Throughput Binding Assay for Estrogen Receptor $\alpha$

As shown in Figure 1, immobilization of the estrogen receptor was observed in  
10 the absence of added antibody, but the binding was too weak to support high throughput screening. Increasing the amount of receptor  $\alpha$  in the well by 13-fold (to 10 pmol) significantly enhanced total counts. However, as a result, the cost of receptor needed for a high throughput screen became prohibitive. To overcome this limitation, the protein target was immobilized to the surface using antibody affinity capture. Use of an antibody  
15 made the assay both more cost-effective and more sensitive. In the experiment shown, total binding was approximately 2200 counts per minute with 0.75 pmol of receptor in the presence of antibody, and was approximately 1800 counts per minute with 10 pmol of receptor in the absence of antibody.

20 All of the antibodies except E51320 enhanced total counts per well significantly over background (receptor + competitor) and over the negative control (no receptor) (Figure 2). However, antibody SRA-1010, which recognizes an epitope at the carboxyl-terminal end of the receptor, produced the largest signal-to-background ratio.

25 The receptor-ligand complex appears to be remarkably stable. After the tracer was added, specific counts dropped slightly after four hours of incubation at room temperature, but the signal-to-background ratio was still over four-fold after 18 hr of

incubation (Figure 3). However, sufficient time is required to form the antibody-receptor complex (Figure 4). Total counts are barely above control when the receptor was added with the tracer instead of with the antibody the previous evening.

5

Saturation binding analysis showed that the affinity ( $K_d$ ) of the tracer ligand for estrogen receptor  $\alpha$  determined by this assay method corresponded to the known affinity of estradiol for its receptor (Figure 5). In addition, dose-dependent competition of tracer binding to the plate-bound receptor with standard estrogens was observed (Figure 6). The  
10 IC<sub>50</sub> for 17 $\beta$ -estradiol over multiple experiments (0.5 to 2 nM) was comparable to those obtained in assays known by those in the art (Korach, K. S., Migliaccio, S. and Davis, V.L. (1995). In Principles of Pharmacology: Basic Concepts and Clinical Applications [Munson, P.L. (Ed)], Chapman and Hall, New York, NY, pp. 809-825; Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. and Gustafsson, J.-  
15 A. (1997). *Endocrinology* 183, 863-870).

### EXAMPLE 3

#### Establishment of a High Throughput Binding Assay for Estrogen Receptor $\beta$

20 To establish that the assay can be applied to other nuclear receptors, essentially the same methodology used to assay the estrogen receptor  $\alpha$  was applied to purified estrogen receptor  $\beta$  using a commercially available polyclonal antibody against it, PA1-313 (Affinity BioReagents). Sheep anti-rabbit immunoglobulin FlashPlates<sup>®</sup> were used for affinity capture of the receptor-antibody complex. As can be seen in Figure 7, 2  $\mu$ g of  
25 the antibody increased the total counts over two-fold relative to non-specific adsorption in the absence of antibody, and increased the signal-to background ratio from 2.5-fold in its absence to six-fold in its presence.

**EXAMPLE 4****Protocol for High Throughput Binding Assay for Estrogen Receptors ( $\alpha$  and  $\beta$ )****Set up Day:**

- 5 Estrogen receptor  $\alpha$ : add 100  $\mu$ L Estrogen Screening Buffer containing 5 mM dithiothreitol, 0.5  $\mu$ g mouse anti-estrogen receptor  $\alpha$  antibody SRA-1010 and 50 ng (0.75 pmol) purified human estrogen receptor  $\alpha$  to each well of a 96 well FlashPlate Plus plate cross-linked with goat anti-mouse antibodies.
- 10 Estrogen receptor  $\beta$ : add 100  $\mu$ L Estrogen Screening Buffer or BGG Buffer containing 2.0  $\mu$ g rabbit anti-estrogen receptor  $\beta$  antibody PA1-313 and 100 ng (1.7 pmol) purified human estrogen receptor  $\beta$  to each well of a 96 well FlashPlate Plus plate cross-linked with sheep anti-rabbit antibodies.
- 15 For either nuclear receptor assay, seal the plate(s) and incubate below 10°C for a minimum of 12 hours.

**Assay Day:**

- Wash the wells three times each with 200  $\mu$ L room temperature phosphate-buffered saline, pH 7.2 using a pipette or automatic washer. Then add 98  $\mu$ L radiolabeled estrogen (0.5 nM, which equals 0.1  $\mu$ Ci of 2000 Ci/mmol iodinated estradiol or 6 nCi of 120 Ci/mmol tritiated estradiol), diluted in Estrogen Screening Buffer + 5 mM dithiothreitol to each well. Then, to the appropriate wells, add 2.5  $\mu$ L of test compound diluted in 30% (v/v) dimethyl sulfoxide/50 mM HEPES pH 7.5. Mix three times by aspiration. Then seal the plate and incubate at room temperature for a minimum of 0.5 hours. Then read each plate for 1 minute in a TopCount scintillation counter, with a 1 minute pre-read delay per plate.
- 20
  - 25

**EXAMPLE 5****Reproducibility of the Assay**

- 5       The coefficients of variation (CV's) across duplicate plates were determined on two separate days, for each assay (Table 1). CV's were 10% or less for both the estrogen receptor  $\alpha$  and the estrogen receptor  $\beta$  assays when they were performed in the presence of the appropriate antibody. With the amounts of receptor used, CV's were higher (14% to over 20%) in the absence of the antibodies. Within an experiment, variability was 1.5 to over three times higher in the absence of antibody compared to in its presence.
- 10       Presumably, this was due to the lower total counts obtained in the absence of the antibodies.

**Table1. Comparison of CV's for the estrogen receptor  $\alpha$  and  $\beta$  assays performed in the absence and presence of antibodies.**

5

<u>Plate Set-Up<sup>a</sup></u>	<u>Average Plate %CV, Day</u>	
	<u>One</u>	<u>Two</u>
hER $\alpha$ , with antibody	10.5	7.12
hER $\alpha$ , without antibody	21.8	13.7
hER $\beta$ , with antibody	10.2	4.20
hER $\beta$ , without antibody	16.1	14.6

<sup>a</sup> Assays were performed as in Figures 2 (human estrogen receptor  $\alpha$ , hER $\alpha$ ) and 7 (hER $\beta$ ), in the presence or absence of the respective antibodies (0.5  $\mu$ g SRA-1010 for hER $\alpha$  and 2  $\mu$ g PA1-313 for hER $\beta$ ). Vehicle (0.75% [v/v] dimethyl sulfoxide, final concentration) was added to all wells. [<sup>3</sup>H]-estradiol was used as the tracer ligand. CV's were calculated for each plate by averaging the column and row CV's. Data from two plates were then averaged.

10

#### REFERENCES

15

1. Cook, N.D. 1996. Scintillation proximity assay: a versatile high-throughput screening technology. *Drug Discovery Today* 1: 287-294.

2. Korach, K.S., Migliaccio, S. and Davis, V.L. 1995. Estrogens, pp. 809-825. In Munson, P.L. (Ed), *Principles of Pharmacology. Basic Concepts and Clinical Applications*, Chapman and Hall, New York, NY.

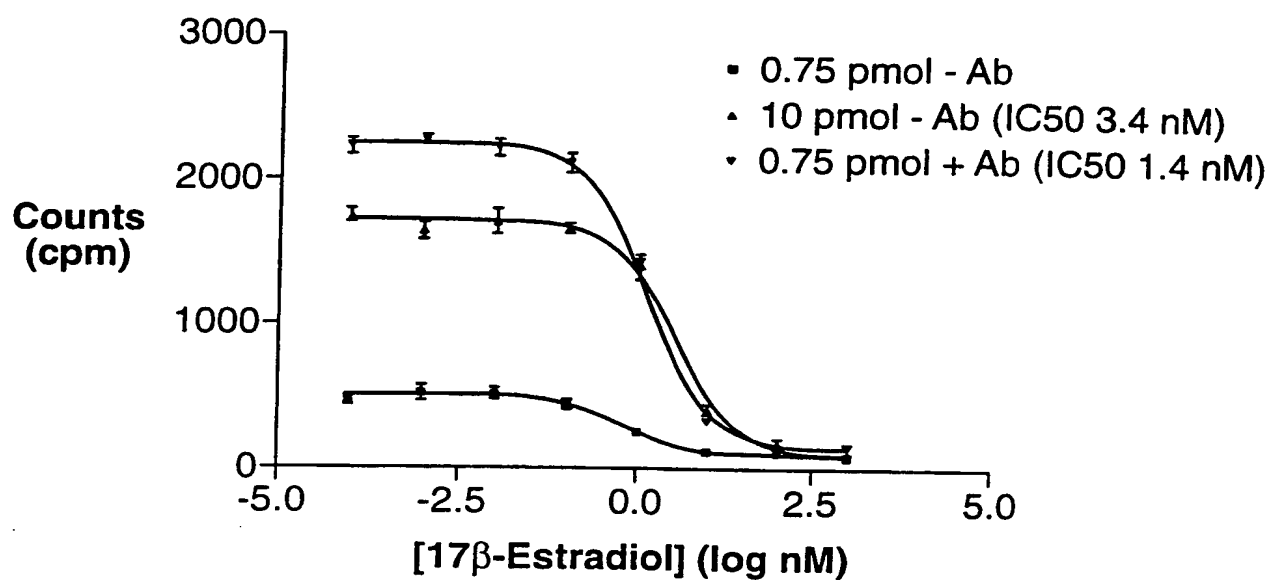
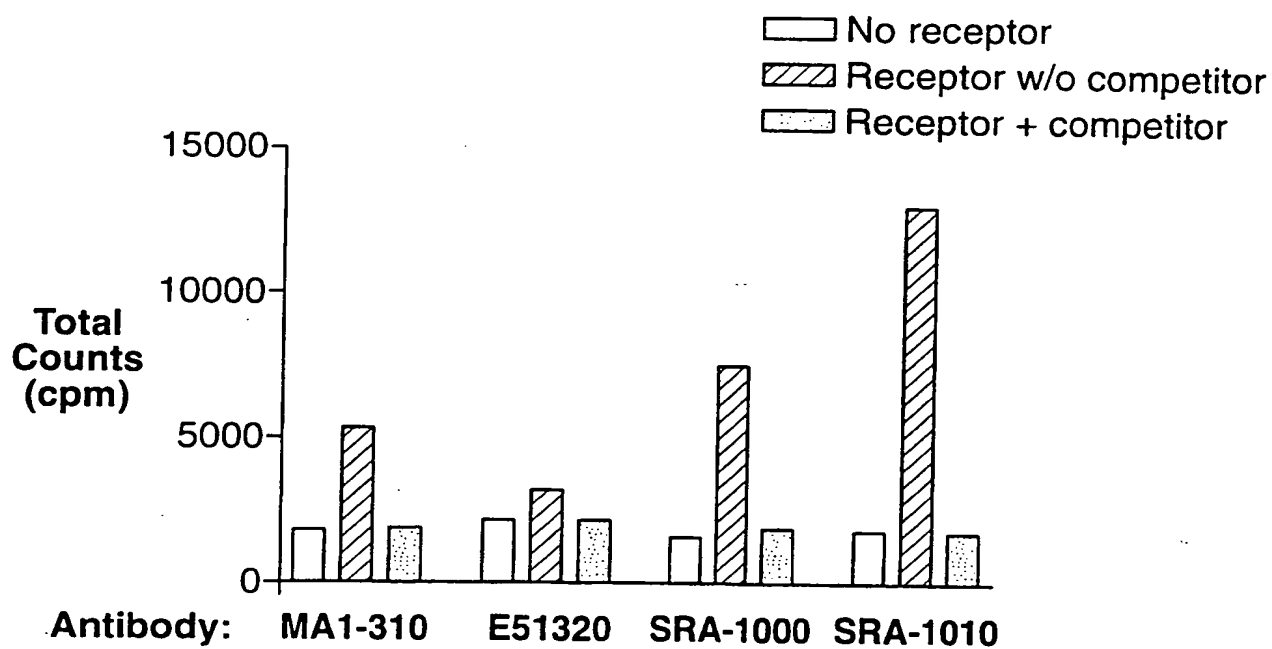


3. Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. and Gustafsson, J.-A. 1997. Comparison of the ligand binding specificity and transcript distribution of estrogen receptors  $\alpha$  and  $\beta$ . *Endocrinology* 183, 863-870.
- 5
4. Picardo, M. and Hughes, K.T. 1997. Scintillation proximity assays, pp. 307-316. In Devlin, J. P. (Ed), *High Throughput Screening*, Dekker, New York, NY.
- 10
5. Tsai, M.-J. and O'Malley, B.W. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* 63, 451-486.
6. Haggblad, J., Carlsson, B., Kivela, P. and Siitari, H. 1995. Scintillating microtitration plates as platform for determination of [ $^3$ H]estradiol binding constants for hER-HBD. *BioTechniques* 18, 146-151.
- 15
7. Elbrecht, A., Chen, Y., Adams, A., Berger, J., Griffin, P., Klatt, T., Zhang, B., Menke, J., Zhou, G., Smith, R.G., Moller, D. E. (1999) L-764406 is a partial agonist of human peroxisome proliferator-activated receptor  $\gamma$ . The role of Cys313 in ligand binding. *J. Biol Chem* 274 (12) 7913-7922.
- 20
8. Nichols, J. S., Parks, D.J. Consler, T. G., Blanchard, S.G. (1998). Development of scintillation proximity assay for peroxisome proliferator-activated receptor  $\gamma$  ligand binding domain. *Anal Biochem* 257(2) 112 – 119.
- 25

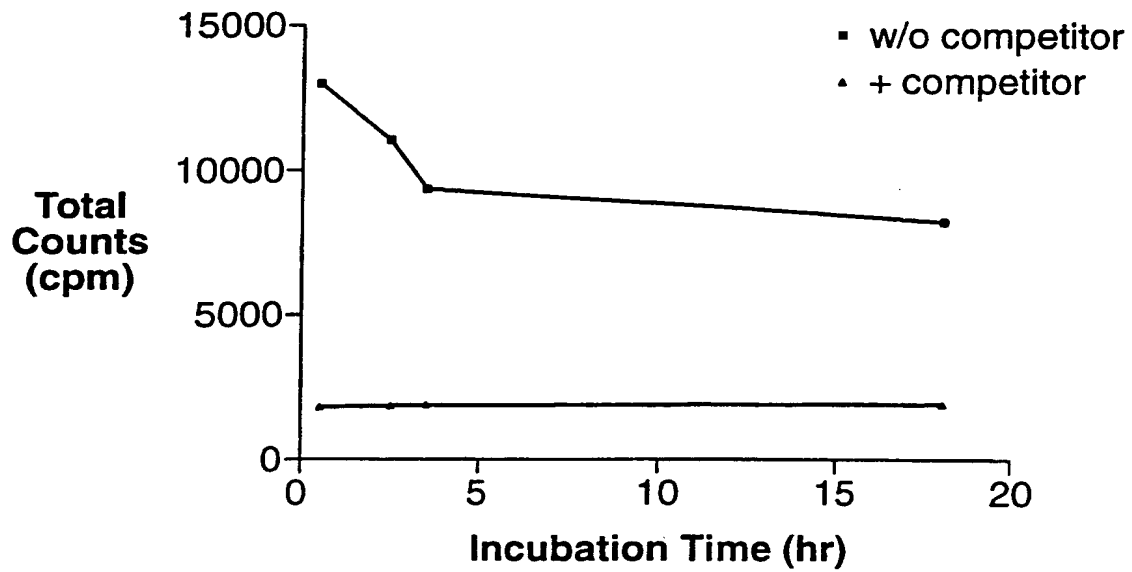
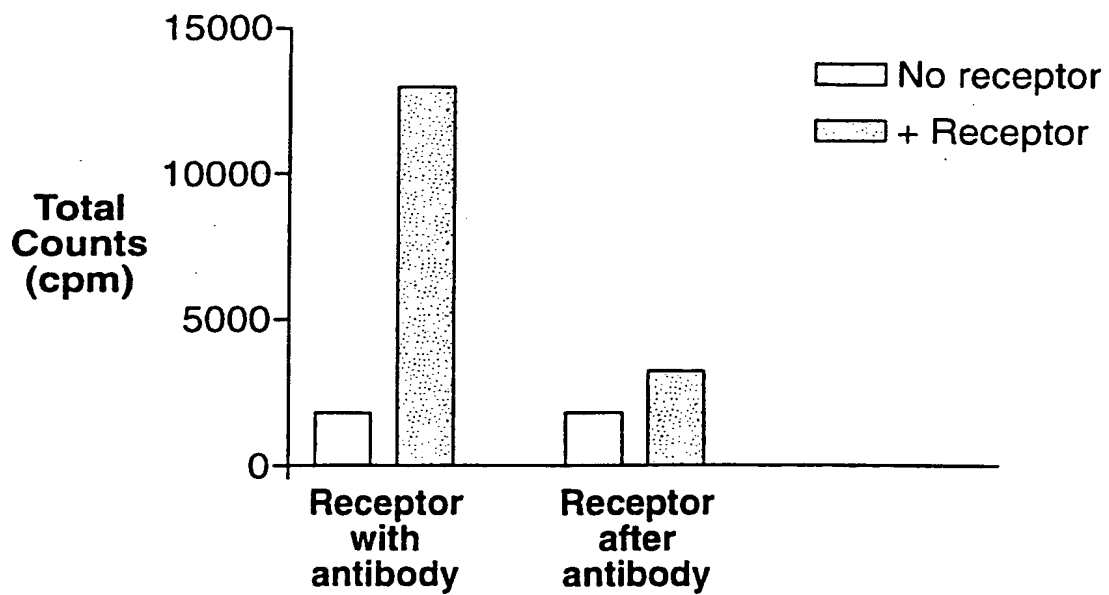
## WHAT IS CLAIMED IS:

1. A method, comprising:
  - a) coating wells of a multi-well, scintillant-coated plate with an anti-species antibody;
  - b) adding to the wells a mixture of an anti-nuclear receptor antibody that also binds to the anti-species antibody, and a nuclear receptor that the anti-nuclear receptor antibody binds;
  - c) adding to the well a mixture of a sample and a labeled ligand that is known to bind to the nuclear receptor; and
  - d) measuring scintillation of the well to determine the amount of labeled ligand bound to the nuclear receptor.
2. The method of claim 1 wherein the nuclear receptor comprises an estrogen  $\alpha$  receptor.
3. The method of claim 1 wherein the nuclear receptor comprises estrogen  $\beta$  receptors.
4. The method of claim 1 wherein the antibody binds specifically to estrogen  $\alpha$  receptors.
5. The method of claim 1 wherein the antibody binds specifically to estrogen  $\beta$  receptors.

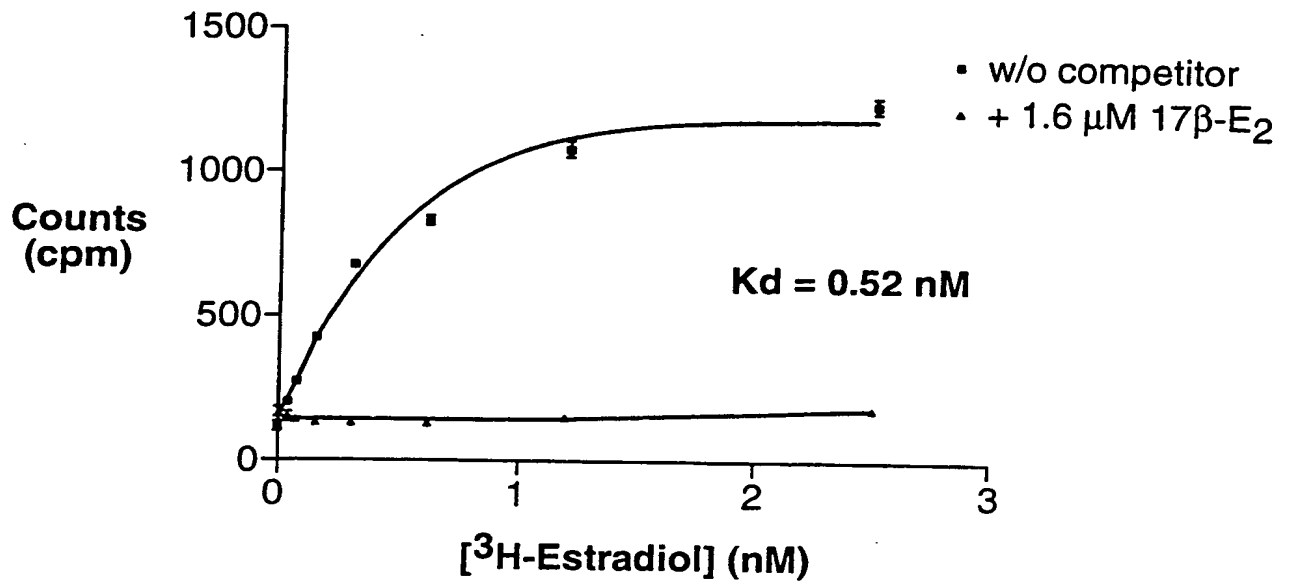
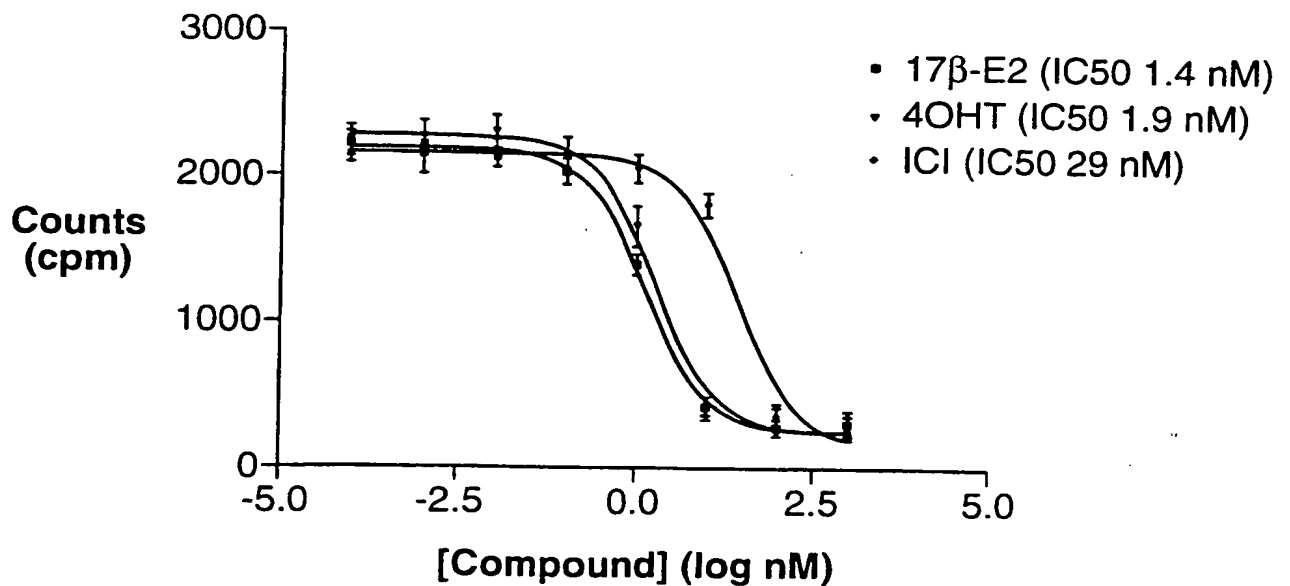
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**FIG. 1****FIG. 2**

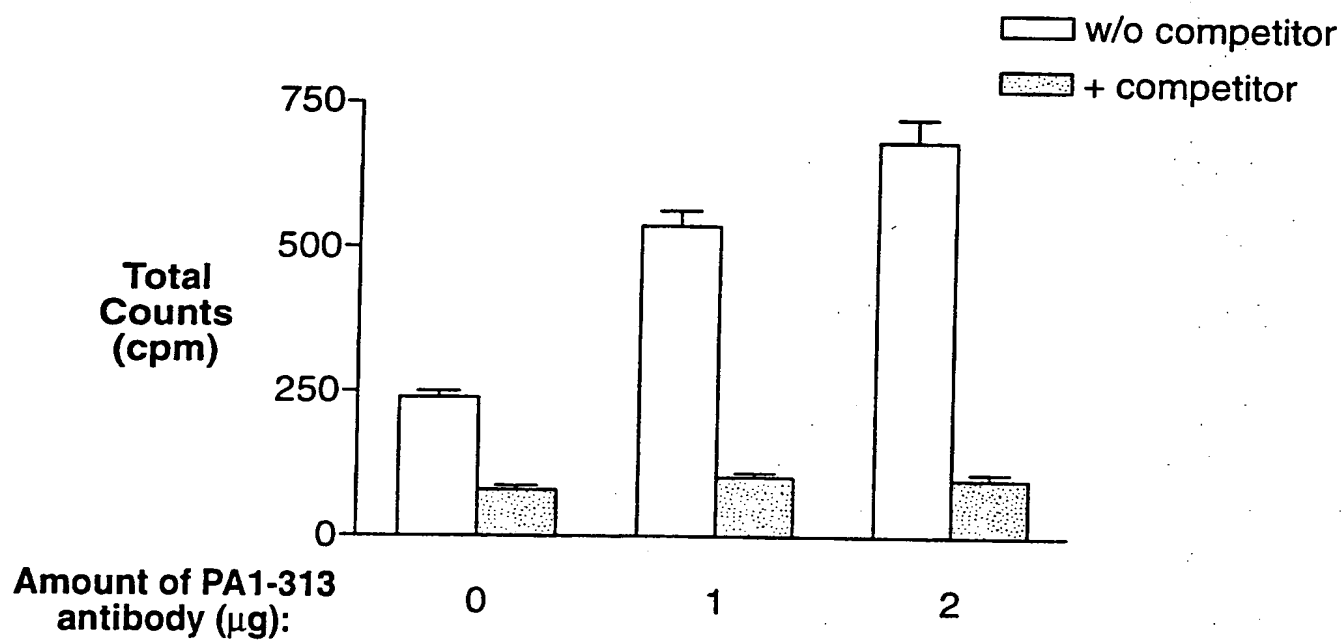
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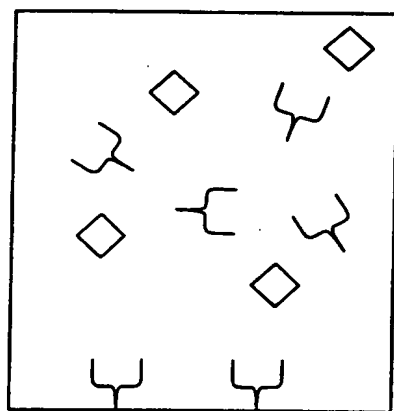
**FIG. 3****FIG. 4**

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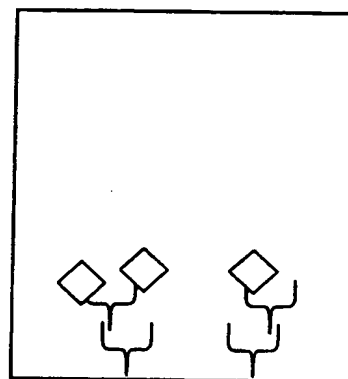
**FIG. 5****FIG. 6**

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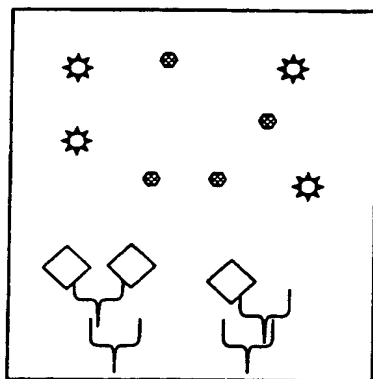
**FIG. 7**

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**FIG. 8**Flow Diagram of the Assay

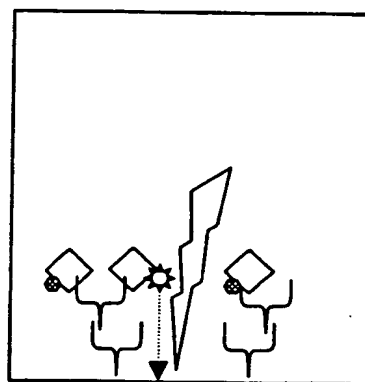
1. Immobilize nuclear receptor via an affinity intermediate (antibody)



2. Wash unbound nuclear receptor and affinity intermediate



3. Add radiolabeled ligand and sample



4. Measure competitive binding of radiolabeled ligand via visible light emission from scintillant

**Legend:**

● Sample ligand

◇ Nuclear receptor

★ Radiolabeled ligand

Y Antibody

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/12863

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST and WEST Patent Database, STN-biosis, caplus, haplus, medline.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,783,398 A(MARCY et al.) 21 July 1998, see entire document.	1-5
Y	US 5,512,429 A (WILTON) 30 April 1996, see full document.	1-5
Y	US 4,568,649 A (BERTOGLIO-MATTE) 04 February 1986, see full document.	2-5
Y	US 5,389,517 A (WOTIZ et al) 14 February 1995, see entire document.	2-5



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 JUNE 2000

Date of mailing of the international search report

26 JUL 2000

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Facsimile No. (703) 305-3230

Authorized officer

LISA V. COOK

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/12863

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C12Q 1/00, 1/44; G01N 31/00, 33/52, 33/53, 33/54, 33/58, 33/566, 33/577;

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/4, 7.1, 7.2, 7.5, 7.72, 7.8, 18, 19, 69.7, 71.2, 320.1, 810, 849, 962, 969, 973, 975; 436/37, 60, 63, 71, 501, 504, 508, 531, 534, 536, 537, 538, 542, 800, 808, 824, 828; 530/324, 344, 350, 415, 812, 816, 827; 536/23.4, 24.1; 930/280; 422/55; 424/1

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

435/4, 7.1, 7.2, 7.5, 7.72, 7.8, 18, 19, 69.7, 71.2, 320.1, 810, 849, 962, 969, 973, 975; 436/37, 60, 63, 71, 501, 504, 508, 531, 534, 536, 537, 538, 542, 800, 808, 824, 828; 530/324, 344, 350, 415, 812, 816, 827; 536/23.4, 24.1; 930/280; 422/55; 424/1